The Role of Hypothalamic Inflammation in Dim Light at Night and High Fat Diet Induced Metabolic Disturbance

Honors Research Thesis

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By

Rebecca A. Lieberman

The Ohio State University
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Project Advisor: Dr. Randy J. Nelson, Professor and Chair, Department of Neuroscience

Abstract

The prevalence of obesity and metabolic disorders is rapidly increasing worldwide. This increase in metabolic dysfunction correlates with increased exposure to artificial light at night (LAN). LAN disrupts the biological clock of living organisms, which controls circadian regulation of energy homeostasis and is important for proper metabolic functioning in humans and animals. Mice housed with dim LAN (dLAN) have previously been reported to develop symptoms of metabolic syndrome. In industrialized societies exposure to LAN and calorie rich diet often occur in tandem and may contribute to the increasing obesity epidemic. Thus, despite the association in humans between light at night and obesity, I have used mice to establish a causative relationship among light at night, high calorie food, and metabolic syndrome. I hypothesized that dLAN combined with a high fat diet (HFD) exacerbates metabolic dysfunction. Fifty four mice were assigned to four groups; (1) standard light-dark (LD) cycle/standard chow, (2) standard LD/HFD, (3) dLAN/standard chow, or (4) dLAN/HFD. After 4 experimental weeks hypothalamic and white adipose tissue was collected for qPCR and immunohistochemical analyses. dLAN and HFD each increased body and fat pad mass compared to LD and standard chow, respectively. Both variables also altered glucose levels and daytime food intake. dLAN and HFD each elevated TNFα and MAC1 in white adipose tissue. However, dLAN did not increase gene expression of TNFα, POMC, or MAC1 in the hypothalamus. These results indicate that central nervous system inflammation is not a primary mechanism driving light induced weight gain. Further understanding of the mechanisms through which dLAN contributes to inflammation and obesity is important for characterizing and treating metabolic disorders.

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1. Introduction

Obesity is a public health problem of global significance and prevalence rates are continually increasing in both affluent Western countries and poor nations all over the world. Health problems associated with obesity are replacing concerns such as under nutrition and infectious disease as the most significant contributors to global ill health (Antipatis, Gill, 2001). Although well-documented factors such as caloric intake, dietary choices, and lack of exercise are known to contribute to the prevalence of obesity and metabolic disorders, additional environmental factors are now considered critical in the development and maintenance of obesity (Hill, Wyatt, Reed, Peters, 2003).

The worldwide increase in obesity and metabolic disorders correlates with increasing exposure to artificial light at night (LAN). The increase in electrical lighting in industrialized countries has allowed people to extend daytime activities into the night and engage in night time shift work. Artificial LAN disrupts the biological clock of living organisms, due to light receptors in the eye that send neural impulses directly to the biological clock (Pauley, 2004). The biological clock controls circadian regulation of energy homeostasis, which has been coupled to proper metabolic functioning, in both humans and nonhuman animals (Lowrey, Takahashi, 2004). This study investigates whether a causal relationship exists between exposure to LAN and obesity by examining the effects of LAN on metabolism of male mice. More specifically, preliminary data suggest that dim light at night (dLAN) and a high fat diet (HFD) each induce obesity, dysregulate glucose metabolism, and increase hypothalamic inflammatory responses. Because of the potential similar mechanisms involved in dLAN and diet induced obesity, it is expected that these results will be amplified when dLAN and HFD are combined.

Central nervous system inflammation can contribute to the development of metabolic diseases and obesity (Hotamisligil, 2006). The hypothalamus regulates glucose

metabolism, food intake, and body mass. Specifically, the mediobasal hypothalamus contains many nuclei related to energy balance. Increased inflammatory responses in this area of the hypothalamus contribute to the development of insulin resistance (Williams, Cutler 2007). Inflammatory responses begin in the central nervous system within hours of consuming high fat food (Thaler et al., 2012). Continued elevation of central nervous system inflammatory responses combined with a long term high fat diet lead to gliosis and damage to proopiomelanocortin neurons (Parton et al., 2007). Importantly, blockade of central inflammatory responses is sufficient to prevent development of insulin resistance and obesity in mice fed a high fat diet (Wisse, Schwartz, 2009). The mechanisms and potential causal relationships among these phenomena are examined.

1.1 Preliminary Studies

Our preliminary studies indicate that mice exposed to either bright or dim light at night significantly increase body mass and reduce glucose processing compared with mice in a standard light-dark cycle, despite equivalent caloric intake and total daily activity. Nocturnal rodents typically eat substantially more food at night; however, dLAN mice shift their feeding pattern so that they consume more than half of their food during the light phase. Restricting food consumption to the active phase in dLAN mice prevents body mass gain. Importantly, these results suggest that low levels of light at night disrupt the timing of food intake and other metabolic signals, leading to excess weight gain (Fonken et al., 2010). Additionally, dLAN mice increase hypothalamic inflammation and elevate insulin levels on a HFD (Fonken et al, unpublished observations). These preliminary data indicate that dLAN in combination with HFD may dysregulate glucose metabolism, increase hypothalamic inflammatory responses, and induce obesity to a greater extent than either of these conditions alone.

2. Materials and Methods

2.1 Experimental Design

Fifty six male Swiss–Webster mice (~8 wk of age) were obtained from Charles River Laboratories for use in this study. The mice were housed individually in propylene cages (30 x 15 x 14 cm) at an ambient temperature of 22 ± 2 °C and provided with filtered tap water *ad libitum.* All experimental procedures were approved by The Ohio State University Institutional Animal Care and Use Committee, and animals were maintained in accordance with the recommendations of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Upon arrival, mice were provided with normal chow and maintained under a 14:10 light/dark (LD) cycle [lights on at 1:00 Eastern Standard Time (EST) ~150 lx] for one week to allow them to adjust to local conditions and recover from the effects of shipping. After the habituation period, mice were randomly assigned a number and placed in one of four groups (n=14 per group); one half were housed in LD conditions and one half were housed in dLAN conditions. Furthermore, within each of these groups mice were randomly placed on a high fat diet (HFD; Research Diets D12451, New Brunswick, NJ, USA) or basic rodent diet (chow; Harlan Teklad 8640, Madison, WI, USA). Mice were weighed during group assignment to confirm that all groups had a similar baseline body mass.

After group assignment, the dLAN mice were transferred to a cabinet with a 14:10 light/dim light cycle; during the light period they were exposed to ~150 lx of light, and during the dim period they were exposed to ~5 lx of light at cage level. The LD mice were transferred to a cabinet with a 14:10 light/dark cycle. Body mass and food intake were monitored weekly. Additionally, the timing of food intake was measured twice daily for 4 consecutive days in the final experimental week. After 4 experimental weeks, 7 mice per

group were used for PCR analysis and the remaining 7 mice per group were used for immunohistochemcal analysis of inflammatory markers.

2.2 PCR

A subset of mice was used to analyze gene expression of TNFα, MAC1, and POMC in the hypothalamus and white adipose tissue. Mice were brought into a procedure room, anesthetized with isoflurane vapors, a blood sample was collected from the retro-orbital sinus, and mice were rapidly decapitated. Brains were removed, placed in RNAlater overnight and then the hypothalamus was dissected out. Epididymal fat pads were removed, weighed, and flash frozen. Total RNA was extracted from hypothalamic and fat tissue using a homogenizer (Ultra-Turrax T8,IKAWorks, Wilmington, NC, USA) and an RNeasy Mini Kit (Qiagen, Austin, TX). RNA was reverse transcribed into complementary DNA with M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Gene expression for POMC, TNFa, and MAC1 was determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Fast Real Time PCR System using Taqman Universal PCR Master Mix. The universal two-step reverse transcriptase PCR cycling conditions used were as follows: 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60° C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve and standardized by comparison to an 18S rRNA signal.

2.3 Immunohistochemistry

To assess histological evidence of inflammatory gliosis in the hypothalamus a fixation- perfusion with PBS followed by 4% paraformaldehyde/PBS was performed. Brains were post-fixed in paraformaldehyde overnight, transferred into 30% sucrose in 0.2M phosphate buffer until permeated, then frozen and stored at −80°C. Using a cryostat,

brains were cut into 40 μm free floating sections and stored at -20°C. The sections were rinsed 3 times in phosphate buffered saline (PBS), slides were blocked (4% BSA in PBS + 0.3% Triton-X) for 1 h at room temperature with constant agitation. Sections were incubated overnight at room temperature with primary rabbit anti-iba-1 (Wako Chemicals, Richmond, VA) diluted 1:1000 in PBS containing 0.4% BSA and 0.025% Triton X-100. The sections were washed four times in PBS. The sections were subsequently incubated for 1 h at room temperature with biotinylated goat-anti-rabbit (Vector Laboratories, Burlingame, CA, USA) 1:1000 in PBS containing 0.4% BSA and 0.025% Triton X-100. Sections were rinsed 4 times in PBS and endogenous peroxidase activity was quenched by incubating for 20 min in methanol containing 3% hydrogen peroxide. After washing three times with PBS, the sections were incubated for 1 hour with avidin-biotin complex (ABC Elite kit, Vector Laboratories). The sections were washed four times in PBS and developed with diaminobenzidine reagent for 90 sec (Sigma, D4168). Following dehydration in a series of graded ethanol dilutions (75%, 95% x 3, 100%), the sections were cleared with xylene and coverslipped using permount. Images were captured on a Nikon E800 microscope at 20X and analyzed using Image J software (NIH).

2.4 Statistical Analysis

The change in body mass over time was analyzed using a repeated measures ANOVA with time as the within-subject factor and diet and lighting condition as the between-subject factors. All other data were analyzed with a two-way ANOVA with diet and lighting condition as the between-subject variables. Following a significant F score, multiple comparisons were conducted with Tukey's HSD test. All analyses were conducted using StatView software (v. 5.0.1, Cary, NC). Results were considered statistically significant if $p \leq 0.05$.

3. Results

3.1 Body Mass

Throughout the 4 experimental weeks, HFD and dLAN each increased body mass in comparison to chow and a LD cycle, respectively (Light: $F_{1,196} = 14.197$, Diet: $F_{1,196} = 65.976$; $p < 0.001$). Mice in all groups had similar baseline body mass ($p > 0.05$), but after the first experimental week both dLAN and HFD elevated body mass (Light: $F_{1,49} = 17.250$, Diet: $F_{1,49} = 50.218$; $p < 0.0001$). At the conclusion of the four experimental weeks body mass gain was elevated by both dLAN and HFD (Light: $F_{1,49} = 15.779$; Diet: $F_{1,49} = 136.447$; $p < 0.001$). Moreover, dLAN-HFD mice increased body mass by 41%, whereas LD mice fed standard chow increased body mass by only 8%. dLAN and HFD each increased epididymal fat pad mass, confirming that the increase in body mass was due to an increase in white adipose tissue (Light: $F_{1,21} = 9.785$, Diet: $F_{1,21} = 103.282$).

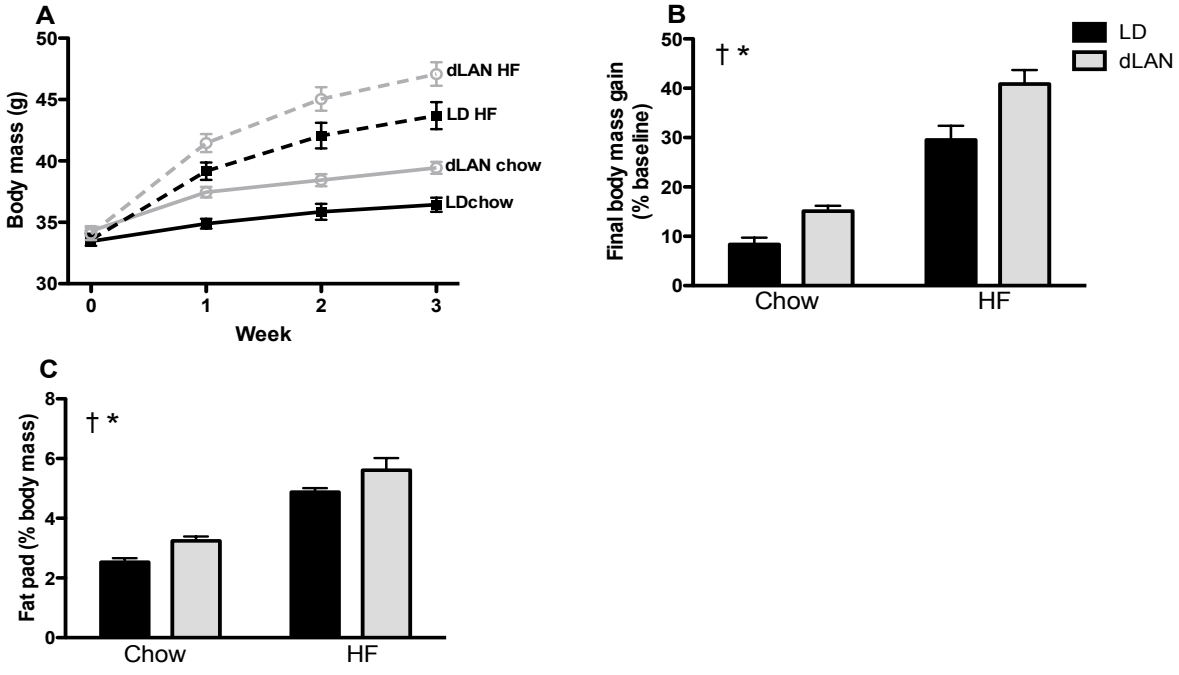

Fig 1. Body mass and fat pad mass were increased in mice exposed to dLAN or a HFD. *(A)* Mice exposed to dLAN or HFD elevated body mass beginning 1 wk after placement in experimental conditions and continuing throughout the remainder of the study. *(C)* Epididymal fat pad mass increased among mice exposed to dLAN or HFD at the conclusion of the study, suggesting increases in body mass may be caused by changes in body fat composition. (†main effect of diet, *indicates main effect of lighting condition).

3.2 Food Intake

Total daily food intake did not differ between lighting conditions ($p > 0.05$). Mice fed a HFD decreased food intake in comparison to the mice fed standard chow because the high fat food was much more calorie dense. HFD and dLAN each resulted in increased daytime food intake as compared to the mice fed chow and exposed to a standard LD cycle, which is atypical for nocturnal rodents (Light: $F_{1,49} = 42.649$; $p < 0.0001$, Diet: $F_{1,49} = 5.509$; $p < 0.05$). The mice exposed to dLAN consumed over half of their food during the daytime as opposed to the nighttime. Additionally, dLAN and HFD each increased blood glucose levels (Light $F_{1,49} = 4.148$, Diet: $F_{1,49} = 5.214$; $p < 0.05$).

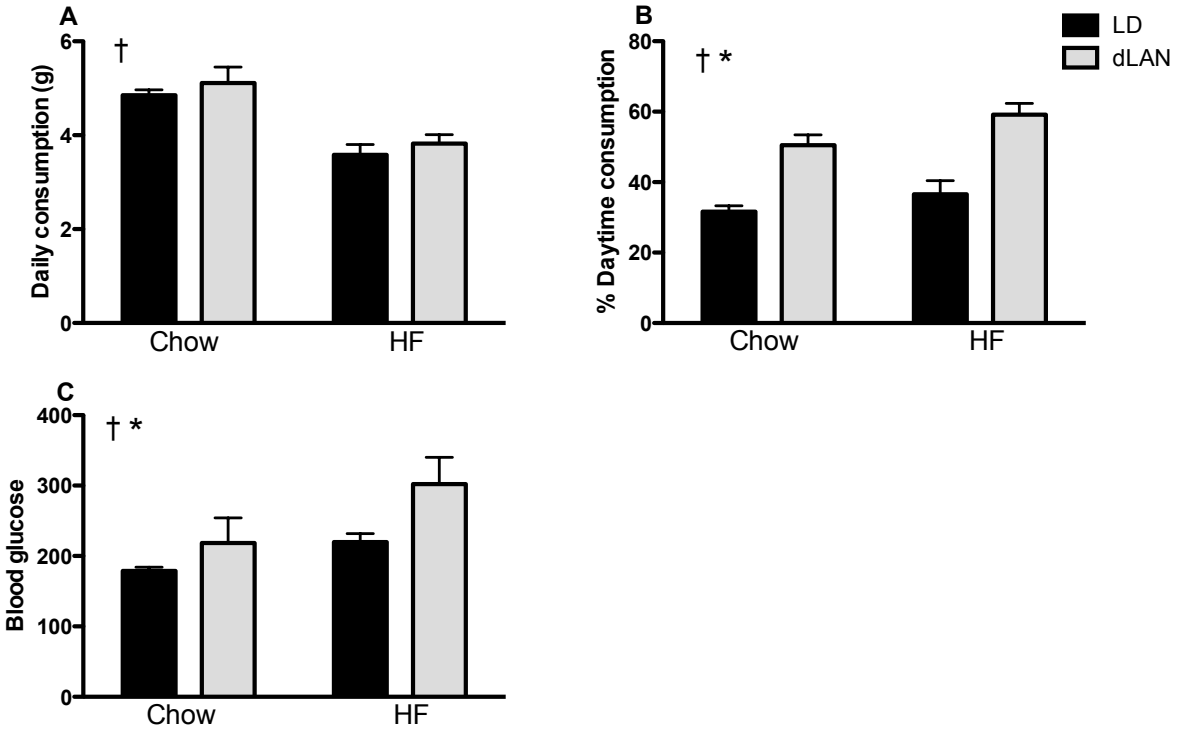

Fig 2. *(A)* Daily consumption did not differ between lighting conditions. Mice in the chow group consumed less grams of food per day because of the decreased calorie content. *(B)* Mice exposed to dLAN and HFD conditions ate more food during the light phase than during the dark phase, behavior that is atypical in nocturnal animals. Mice in these groups also increased blood glucose levels *(C).* (†main effect of diet, *indicates main effect of lighting condition).

3.3 Hypothalamic Inflammation

HFD elevated gene expression of TNFα relative to standard chow in the hypothalamus as was expected ($F_{1,21} = 4.433$; $p < 0.05$). Additionally, HFD elevated the concentration of Iba1 positive cells in the arcuate nucleus of the hypothalamus ($F_{1,21} = 9.612$; $p < 0.01$). dLAN increased the Iba1 positive cell concentration in the chow group, but had no effect in the HFD group. MAC1 gene expression was not affected by diet or light ($p > 0.05$), which may be due to the distribution of hypothalamic microglia. Additionally loss of POMC neurons is a predicate of metabolic disease; however, gene expression was not affected by diet or light ($p > 0.05$, data not shown).

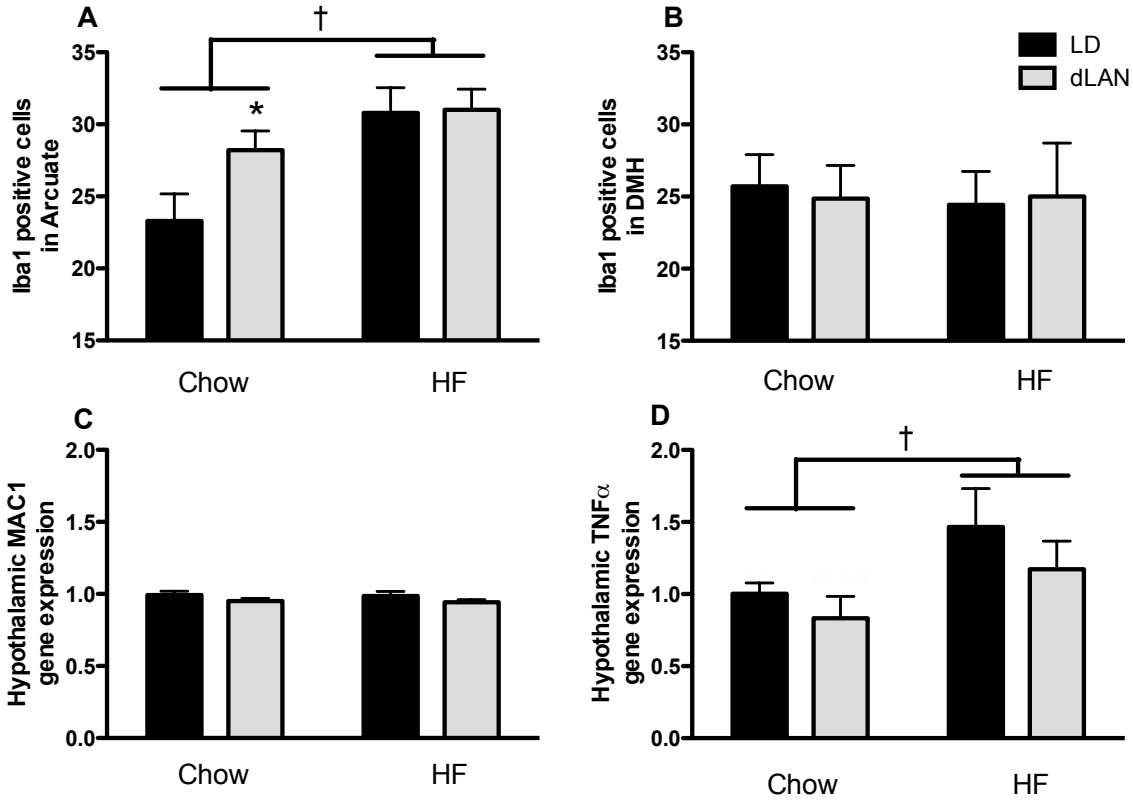

Fig 3. *(A)* HFD elevated the number of Iba1 positive cells in the arcuate nucleus. There was no effect of dLAN on Iba1 positive cells among HFD mice; however, dLAN increased the number of Iba1 positive cells within the chow group. *(B, C)* No differences in Iba1 positive cells in the DMH or MAC1 expression were apparent between light or dietary conditions. This may reflect the distribution of hypothalamic microglia. *(D)* HFD elevated hypothalamic TNFα expression, but there was no effect of light. (†main effect of diet, *indicates main effect of lighting condition).

3.4 Peripheral Inflammation

Levels of pro-inflammatory cytokines were evaluated in the white adipose tissue (WAT) and liver, as chronic low level inflammation results from obesity in peripheral tissues (Gregor, Hotamisligil, 2011). In the WAT, dLAN and HFD each elevated gene expression of MAC1, indicating increased macrophage infiltration. (Light: $F_{1,20} = 9.304$, Diet: $F_{1,20} = 25.442$; $p < 0.01$). Additionally, dLAN and HFD elevated expression of the proinflammatory cytokine TNFα (Light: $F_{1,20} = 4.649$, Diet: $F_{1,20} = 4.979$; $p < 0.05$). However, diet and lighting condition did not effect gene expression in the liver ($p > 0.05$, data not shown). Previous research states that changes in peripheral inflammation often take longer than 4 weeks to develop in response to HFD (Kim et al., 2008).

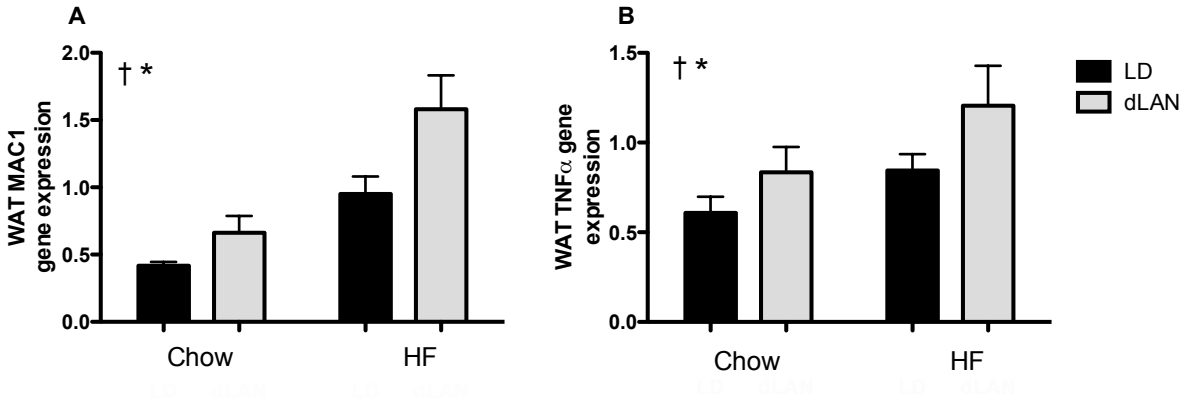

Fig 4. *(A)* In the white adipose tissue, HFD and dLAN each increased gene expression of MAC1, indicative of macrophage infiltration. *(B)* These factors also increased TNFα, a proinflammatory cytokine whose levels correlate with the degree of adiposity and insulin resistance. (†main effect of diet, *indicates main effect of lighting condition).

4. Discussion

This experiment was designed to test the effects that dLAN and HFD have on metabolic dysfunction when combined, as they each induce metabolic dysfunction individually. We hypothesized that similar mechanisms would be responsible for dLAN and HFD induced obesity. Furthermore, we predicted that dLAN and HFD would exacerbate metabolic dysfunction when combined, as evidenced by increased body mass gain, altered

timing of food intake, and peripheral and hypothalamic inflammation.

Diet and lighting condition each influenced body mass gain on a similar time course. HFD and dLAN each increased weight gain after only the first experimental week and continued to do so for each of the remaining weeks. Moreover, dLAN significantly contributed to weight gain in both of the groups and its effects were not masked by a HFD. Increased body mass gain results from being exposed to as little as ~5 lx of light during the nighttime as opposed to complete darkness. Additionally, dLAN and HFD increase the amount of daytime food intake, which is atypical for nocturnal rodents (Arble et al., 2009). The mice exposed to dLAN and a HFD consumed a majority, or 59.14%, of their food during the daytime. Metabolism and the circadian clock are intrinsically linked and metabolic abnormalities occur when timing of food intake is altered (Arble et al., 2009). This suggests that weight gain due to dLAN or HFD may be a consequence of altering the time of food consumption. Importantly, blocking access to food during the daytime hours can prevent increases in weight gain due to a HFD or dLAN (Hatori et al., 2012, Fonken et al., 2010).

The effects that LAN has on metabolic inflammation have not been previously characterized. In metabolically related peripheral tissues obesity leads to chronic low level inflammation (Gregor, Hotamisligil, 2011). I report that dLAN increased inflammation in the white adipose tissue. dLAN and HFD each increased gene expression of TNFα and MAC1. Levels of TNFα correlate with degree of adiposity and insulin resistance (Kollias, Sfikakis, 2010). Moreover, mice lacking TNFα or its receptors show protection against developing insulin resistance (Vazquez, 2008). Increased MAC1 gene expression is indicative of macrophage infiltration of adipose tissue and is present in obese mice as expanding adipocytes produce chemotactic signals that lead to macrophage recruitment (Hotamisligil, 2006). Although dLAN produces inflammation in the WAT that is characteristic of obesity, peripheral inflammation generally follows the onset of obesity,

and is not the primary mechanism driving weight gain.

In contrast, inflammation of the central nervous system can contribute to the development of metabolic diseases and obesity as hypothalamic inflammation is apparent 24 hours after the onset of a HFD (Thaler, 2012). Moreover, the blockade of central inflammatory responses is sufficient to prevent development of insulin resistance and obesity in mice fed a HFD (Wisse, Schwartz, 2009). However, our data does not support the hypothesis that LAN induces obesity through a similar mechanism involving hypothalamic inflammation. HFD elevated gene expression of TNFα in the hypothalamus, but there was no effect of lighting condition. This could result from the time course as a previous study on rodent models revealed a complex on-off-on pattern of elevated gene expression of TNFα in the hypothalamus (Thaler, 2012). Neither light nor diet had an effect on MAC1 gene expression. This may be due to the lack of specificity of the hypothalamic area, as HFD increased the number of Iba-1 positive cells in the arcuate nucleus of the hypothalamus but not in other nuclei such as the dorsal medial hypothalamus. dLAN increased the number of Iba1 positive cells in the chow group, but not in the HFD group. There may be a ceiling effect that represents the maximum amount of microglia that can infiltrate the arcuate nucleus as a result of metabolic dysfunction.

POMC cells play an important role in protecting against obesity and loss of these cells is sufficient to cause excess weight gain in mice (Thaler, 2012). However, neither light nor diet had an effect on POMC gene expression, possibly because of the time course of the study. A study that showed a 25% reduction in hypothalamic POMC cells in mice fed a HFD was over an 8 month period. There may be a fundamental difference between the hypothalamic inflammation that results as a neuroprotective response at the start of a HFD and that associated with chronic HFD exposure (Thaler, 2012). The lack of elevated gene expression of TNFα, POMC, MAC1 and Iba1 in mice exposed to dLAN suggests that central

nervous system inflammation is not a primary mechanism driving dLAN induced weight gain.

This study shows that calorie rich diet combined with nighttime light exposure increases body mass gain and peripheral inflammation. Importantly, central nervous system inflammation appears not to be the primary mechanism for light induced weight gain. Due to the significant amounts of nighttime light exposure in industrialized societies via light pollution, shift work, and technology use, further understanding of the mechanisms through which LAN contributes to inflammation and obesity is important in the treatment of metabolic disorders.

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